VIROLOGY 243, 229–234 (1998)
ARTICLE NO. VY989052

Retroviral Recombination Is Nonrandom and Sequence Dependent

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Received January 15, 1998; accepted January 23, 1998

Sequence variation plays a significant role in the pathogenesis and persistence of retroviral infections and is a major obstacle in the development of vaccines as well as therapies against lethal diseases caused by retroviruses. Recombination is one means by which sequence variation is generated. However, the basic molecular mechanisms of recombination are not adequately understood. In the present study, a spleen necrosis virus (SNV) recombination system was used to ask whether a known hot spot for mutation was also a hot spot for retroviral recombination. The system consisted of a pair of SNV vectors expressing two drug-resistance genes, constructed so that recombinants could be selected by a double resistant phenotype. Restriction enzyme site differences engineered into the vectors were used to map the location of recombination sites within relatively small intervals (55 to 420 bp). The vectors were modified to create two pairs that differed only by the presence of runs of identical nucleotides. The runs of identical nucleotides had been shown previously to be hot spots for frameshift mutations during SNV reverse transcription. Each vector pair was introduced into DSDh helper cells by infection. Viruses were harvested from doubly infected DSDh helper cells and used to infect D-17 target cells. Proviral sequences from 228 cell clones were analyzed by polymerase chain reaction and restriction enzyme digestion. Significant differences in the patterns of recombination were found between the two pairs of vectors. In particular, the frequency of recombination was higher than expected in the interval immediately following the runs. For both pairs of vectors, the overall pattern of recombination was nonrandom and one region was refractory toward recombination.

INTRODUCTION

Retroviruses replicate by an error-prone process called reverse transcription in which their single-stranded, positive-sense RNA genome is converted to a proviral DNA intermediate (Dougherty and Temin, 1986; Preston *et al.,* 1988; Roberts *et al.,* 1988; Temin, 1976). Reverse transcriptase does not have a 3′ to 5′ exonuclease activity to ensure genomic accuracy during polymerization (Battula and Loeb, 1976; Morrison *et al.,* 1991; Roberts *et al.,* 1988). Also, cellular repair systems are not available to the virus during reverse transcription. Retroviruses further increase levels of genomic variation by recombining their two RNA molecules during reverse transcription (Hu and Temin, 1990a).

The primary nucleic acid sequence is known to affect the type and rate of mutation during reverse transcription. For example, runs of identical nucleotides are hot spots for frameshift mutations *in vivo* (Burns and Temin, 1994; Pathak and Temin, 1990b), while direct repeat sequences are hot spots for deletion mutations (Pathak and Temin, 1990a; Pulsinelli and Temin, 1991). Another example is the GpA dinucleotide, which is a hot spot for G to A transition mutations (Vartanian *et al.,* 1991). Finally, the context of the primary sequence may also affect mutation rates for certain sequence motifs (Kunkel, 1990).

Beyond the level of the primary sequence, secondary structure may also affect the process of mutation. Open regions of viral RNA (regions not involved in stable base-paired interactions) have been found to correlate with variable regions of human immunodeficiency virus type 1 (HIV-1) (Le *et al.,* 1989) and murine leukemia virus (Parthasarathi *et al.,* 1995). The open regions may have been more susceptible to certain types of genetic damage (such as breaks) that resulted in the observed genomic variation (Le *et al.,* 1989; Parthasarathi *et al.,* 1995).

In contrast to the mechanism of mutation, much less is known about sequences and structures that promote retroviral recombination. It has been reported that HIV-1 reverse transcriptase terminates synthesis within hairpin stem structures *in vitro* (DeStefano *et al.,* 1992a), suggesting that closed regions may promote recombination. Mikkelsen *et al.* (1996) identified a recombination hot spot in a murine leukemia virus system. The hot spot consisted of a 33-bp region containing RNA dimerization and packaging sequences, a region known to contain stem-loop structures required for encapsidation of the viral RNA (Yang and Temin, 1994).

The hypothesis for the present study is that runs of identical nucleotides serve as hot spots for retroviral recombination. This hypothesis was justified by several reports in the literature. First, runs of identical nucleo-

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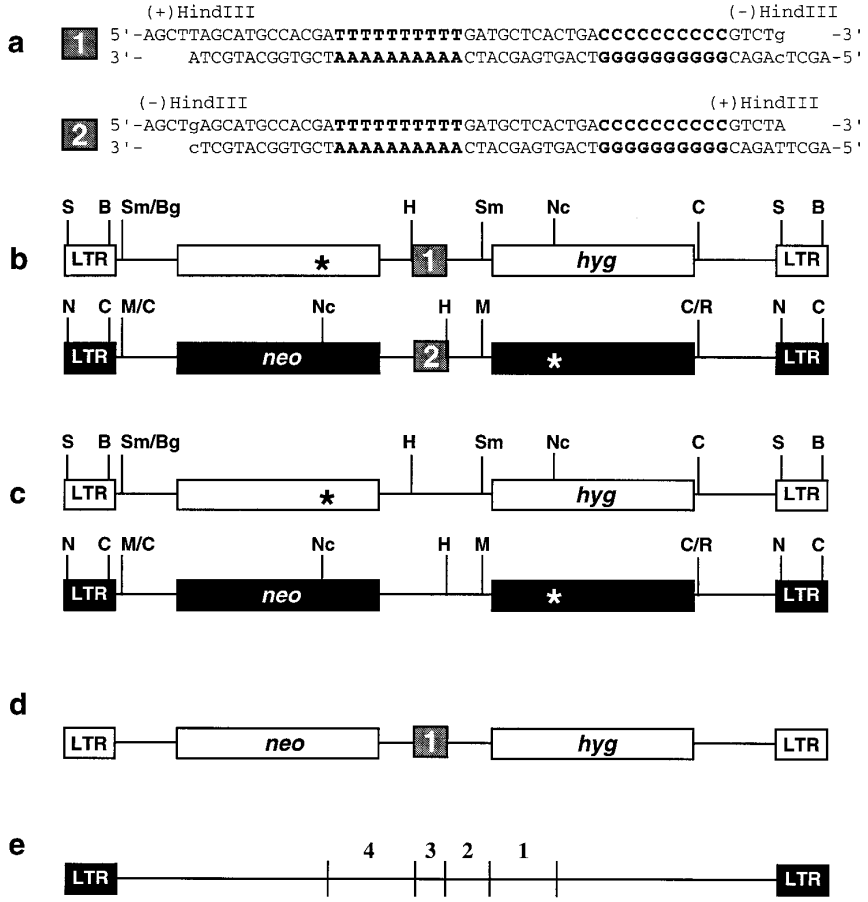

FIG. 1. Insert sequences and vectors. (a) Long synthetic oligonucleotides containing runs of identical bases (bold) were annealed to create inserts 1 and 2. Sticky ends are compatible with the restriction enzyme *Hin*dIII. The righthand *Hin*dIII site of insert 1 is nonfunctional due to an internal A to G base change that was engineered into the oligonucleotide. Likewise, the lefthand *Hin*dIII site of insert 2 is nonfunctional due to a T to G base change. Lowercase letters indicate base changes. Two pairs of vectors that either contain (b) or do not contain (c) runs of identical nucleotides are shown. Each vector contains the neomycin and hygromycin resistance genes; however, one of the two genes in each vector is nonfunctional due to a frameshift mutation (*). Vectors differ by restriction enzyme sites and are distinguished by open white or solid black boxes. Restriction enzymes are as follows: N, *Not*I; C, *Cla*I; M, *Mlu*I; Nc, *Nco*I; H, *Hin*dIII; R, *Eco*RI; S, *Sac*I; B, *BamH*I; Sm, *Sma*I; Bg, *Bgl*II. The gray-shaded boxes numbered 1 and 2 represent the oligonucleotide inserts containing runs of identical nucleotides. (d) Control vector. (e) Recombination intervals.

tides had been identified previously as hot spots for frameshift mutations, and recombination was a potential mechanism for the frameshift mutations (Burns and Temin, 1994; Pathak and Temin, 1990b). Second, identical nucleotide runs were shown to serve as pause and termination sites for HIV-1 reverse transcriptase *in vitro* (DeStefano *et al.,* 1992a; Klarmann *et al.,* 1993). Third, pause sites were shown to enhance recombination by HIV-1 reverse transcriptase *in vitro* (DeStefano *et al.,* 1992b).

To test the hypothesis, a spleen necrosis virus (SNV)-based recombination system was used to study and compare recombination events for pairs of retroviral vectors that differed only in the presence of identical nucleotide runs. Recombination events were nonrandom for both pairs of vectors. The insertion of the identical nucleotide runs significantly altered the location of recombination.

RESULTS

Design of vectors

The SNV recombination system consisted of two retroviral vectors (Fig. 1). Each of the two vectors contained genes for neomycin and hygromycin resistance. However, each vector had one of the genes disabled by a frameshift mutation so that recombinants could be selected by a double resistant (double^r) phenotype. Two pairs of vectors were constructed. One pair contained runs of identical nucleotides between *neo* and *hyg* (Figs. 1a and 1b), and the other pair contained nonrun sequences at the same location (Fig. 1c). The run-containing inserts were similar to ones used by Burns and Temin (1994). A control vector expressing functional *neo* and *hyg* genes was constructed to test for expression of *hyg* after insertion of the run-containing insert (Fig. 1d).

Four intervals, defined by the locations of frameshift

mutations in *neo* and *hyg* and by restriction enzymes *Hin*dIII, *Sma*I, and *Mlu*I, were studied (Fig. 1e). Intervals were numbered one through four in the right to left direction to correspond with first-strand synthesis by reverse transcriptase.

Analysis of doubly resistant helper cell clones

Double^r helper cell clones were analyzed for proviral structure, insert sequence, and virus production. Proviral sequences were amplified by polymerase chain reaction (PCR), cut with various restriction enzymes listed in Fig. 1, and analyzed by agarose gel electrophoresis. Comparison of the patterns and sizes of the DNA fragments revealed that all cell clones contained two different proviral sequences as expected (1 white and 1 black; Fig. 1).

After confirming the integrity of the overall proviral structures, the sequences of the inserts were then determined. The individual proviruses were amplified by PCR, and the products were directly sequenced. Sequence analysis revealed that all cell clones contained the appropriate insert sequences.

Control experiment

Recombinant proviruses isolated from doubly resistant target cell clones had to express functional *neo* and *hyg* genes from the same retroviral genome. In order to rule out the possibility that insertion of the run-containing oligonucleotide affected splicing and expression of the downstream *hyg* gene, a control vector was made which contained insert 1 between functional *neo* and *hyg* genes (Fig. 1d). Vector DNA was transfected into DSDh helper cells, and hygromycin B was added to the medium to select for cells harboring integrated vector sequences. Virus was harvested from the transfected cells and was used to infect D-17 target cells. In each experiment, constructs were tested in parallel using a single drug selection protocol, and the G418-resistant (G418^r) and hygromycin-resistant (Hyg^r) titers were compared. Results from these experiments showed that the G418^r/Hyg^r ratio was virtually identical for pJD216NeoHy (original vector) and pJD216NeoHy+Insert (data not shown). Therefore, insertion of the run-containing oligonucleotide did not affect expression of the downstream *hyg* gene from a vector with two functional drug resistance genes.

Recombination rate

The recombination rates were determined by harvesting virus from doubly infected helper cell clones, infecting D-17 target cells, determining viral titers, and calculating the rates (Fig. 2 and Materials and Methods). The mean recombination rate for the run-containing cell clones was 2.3 ± 1.2%/kb per cycle with a range of 0.67 to 4.1%. For the nonrun cell clones the rate was 3.6 ± 1.5%/kb per cycle, with a range of 1.6 to 6.2%.

An analysis of variance (ANOVA) was conducted using a nested design and mixed procedure in the Statistical Analysis System (SAS) Version 6.12. The analysis accounted for variability within individual cell clones in the run and nonrun groups. Comparison of the rates for the run and nonrun groups revealed that the difference was not statistically significant ($P = 0.1074$). However, recombination rates for the nonrun group generally tended to be higher.

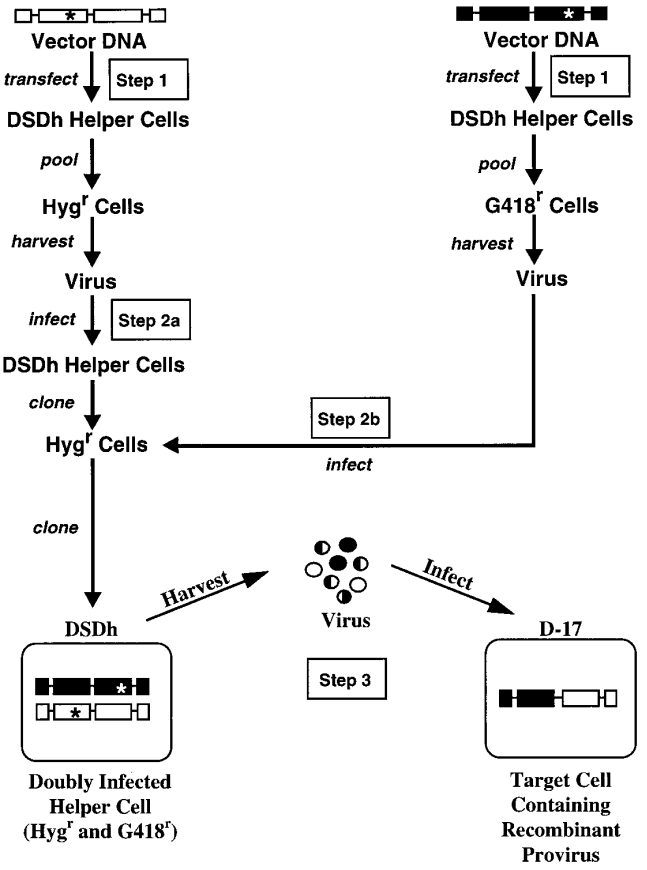

FIG. 2. Establishment of doubly infected cell clones and protocol for one cycle of replication. In step 1, each vector DNA was transfected separately into DSDh helper cells. Selection with the appropriate drug was performed, and resistant cells were pooled separately. Virus was harvested from hygromycin resistant (Hyg^r) step 1 cells and was used to infect fresh DSDh helper cells (step 2a). Hyg^r cells were cloned and were then infected with virus harvested from G418-resistant (G418^r) step 1 cells (step 2b). Finally, selection with hygromycin and G418 was performed, and doubly resistant cell clones were isolated. Virus was isolated from doubly infected helper cell clones and was used to infect D-17 target cells in order to achieve one cycle of replication (step 3).

Location of recombination sites

Proviral sequences from doubly resistant D-17 cell clones were amplified by PCR and analyzed by restriction enzymes *Hin*dIII, *Mlu*I, and *Sma*I. The patterns of bands observed on the agarose gels revealed the locations of the recombination events. A total of 86 run-containing clones and 142 nonrun-containing clones were analyzed. The number of recombination events

TABLE 1

Frequency of Recombination Events

	No. (%) of recombination events in vector intervals				
	4	3	2	1	Total
Run					
Observed	66 (77)	2 (2)	15 (17)	3 (4)	86 (100)
Expected[a]	35 (41)	4 (5)	15 (17)	32 (37)	86 (100)
Nonrun					
Observed	67 (47)	22 (16)	29 (20)	24 (17)	142 (100)
Expected[a]	57 (40)	11 (8)	23 (16)	51 (36)	142 (100)

[a] Expected No. = (length of interval ÷ total length of all intervals) (No. clones).

observed within each interval was counted and compared with the expected number. The expected number reflects random recombination within the set length of the interval (Table 1).

Frequencies were used in χ^2 analysis and Fisher's exact test to determine if differences were statistically significant; Fisher's test was used to analyze those intervals with small frequencies (<20). The observed number of recombination events in the run-containing clones was significantly higher than expected for interval 4 ($P < 0.005$), while for the nonrun-containing clones the number was significantly higher than expected for interval 3 ($P < 0.05$). For both the run- and nonrun-containing clones, the observed number of recombination events for interval 1 was significantly lower than expected ($P < 0.001$ and $P < 0.005$, respectively). Finally, the overall pattern of recombination for both pairs of vectors was nonrandom ($P < 0.005$) (Table 1).

DISCUSSION

In the studies described in this report, the rate of recombination and the location of recombination was determined for two pairs of SNV vectors that differed only by the presence of runs of identical nucleotides. The identical nucleotide runs had been shown previously to be hot spots for frameshift mutation during SNV reverse transcription (Burns and Temin, 1994; Pathak and Temin, 1990b). The pattern of recombination was significantly altered between the two pairs of vectors.

Recombination rates were 2.3%/kb per cycle for run-containing vectors and 3.6%/kb per cycle for the nonrun vectors. Overall, the rates and variations were consistent with previous reports on this system (Hu and Temin, 1990a,b, 1992; Jones *et al.,* 1994a,b, 1993). It was not surprising that rate differences were not statistically significant. Previous studies have shown that despite some rather drastic treatments and changes in the SNV vector system, recombination rates were not altered. For example, exposing the virions to gamma irradiation (Hu and Temin, 1992), altering the location of the dimer linkage site (Jones *et al.,* 1993), and copackaging different sized retroviral genomic RNAs (Jones *et al.,* 1994b) did not significantly alter the rate of recombination.

However, a surprising result was that the frequency of recombination was significantly higher in interval 4 immediately following the runs (Fig. 1, Table 1). A change in the location of recombination was anticipated, but it was expected that the change would be a higher frequency in interval 3, which contained the runs (Fig. 1, Table 1). Instead, the runs appeared to exert their influence on recombination by acting at a distance (within 420 bp). It is possible that the deletion of certain sequences was responsible for the altered pattern of recombination. During construction, 89 bp of vector sequence was replaced with a 55-bp oligonucleotide insert containing the runs. Alternatively, RNA structure may explain the altered pattern of recombination.

Another remarkable result was that recombination in interval 1 (Fig. 1, Table 1) appeared to be inhibited. Frequencies of recombination for this interval were lower than expected (Table 1). Since this inhibitory effect was observed for both pairs of vectors, it was not a run-related phenomenon. However, the inhibitory effect was more pronounced in the run-containing clones.

In conclusion, this study shows that while overall recombination rates are fairly stable for a given retrovirus system, relatively small sequence changes (less than 100 bp) can dramatically alter the pattern of recombination. Such information is extremely valuable in understanding the basic molecular mechanisms of recombination and in understanding the natural patterns of retroviral variation observed worldwide. These findings are also helpful in the design of retroviral-based gene therapies. For example, the information could be used to avoid unwanted gene rearrangements in retroviral vectors used for therapies or vaccines. In the future, additional research should be directed toward understanding higher order molecular structures and their affects on the process of retroviral recombination.

MATERIALS AND METHODS

Construction of vectors

Long oligonucleotides (54-mers) were synthesized, 5′-phosphorylated with T4 polynucleotide kinase, and annealed to create double-stranded DNA inserts 1 and 2 (Fig. 1a). The inserts were ligated between two *Hin*dIII sites in pWH13 and pWH204 (Hu and Temin, 1990b) to create vectors pDB13TC10 (white) and pDB204TC10 (black) (Fig. 1b). The 54-bp inserts were cloned between *neo* and *hyg,* replacing 89 bp of sequence. As a result of a base change engineered at the end of each insert, one of two *Hin*dIII sites for each insert is nonfunctional after cloning. The location of the functional *Hin*dIII site differed for each vector in order to map recombination sites.

A pair of vectors was constructed that did not contain runs of identical nucleotides for comparison of rates and locations of recombination (Fig. 1c). One of two *Hin*dIII sites in pWH13 and pWH204 (Hu and Temin, 1990b) was disabled by partial digestion with *Hin*dIII and subsequent fill-in and ligation to create vectors pWH13XH2 (white) and pWH204XH1 (black) (Fig. 1c). The partial digestions were performed by using 0.5 U of *Hin*dIII/μg of DNA at 37°C for 45 min. The digestion was stopped by addition of EDTA to a final concentration of 15 mM and incubation at 65°C for 20 min. Fill-in reactions were performed using T4 DNA polymerase under manufacturer's conditions (New England Biolabs, Beverly, MA).

The control vector was constructed by cloning insert 1 (Fig. 1a) between two *Hin*dIII sites of a previously characterized vector named pJD216NeoHy (Dougherty and Temin, 1986).

Ligation mixtures were used to transform bacterial strain NM522 of *Escherichia coli.* Small-scale DNA preparations from individual bacterial colonies were screened by restriction enzyme analysis and the final clones were selected by DNA sequencing.

Cells

DSDh and D-17 cell lines were maintained in Temin-modified Eagle's medium (Biologos, Naperville, IL) (Temin, 1968) supplemented with 6% calf serum (EC_6) at 37°C and 5% CO_2. The DSDh helper cell line (No. CRL 2131, American Type Culture Collection, Rockville, MD) expresses the *gag-pol* and *env* genes of SNV and was derived from D-17 cells (Hu and Temin, 1990a). The D-17 cell line (No. CCL 183, American Type Culture Collection) was derived from a dog osteosarcoma and is permissive for SNV infection (Riggs *et al.,* 1974). Selection with hygromycin B (Calbiochem-Novabiochem Corp., La Jolla, CA) was performed at 80 units/ml. Selection with G418 sulfate (a neomycin analog; Gibco BRL, Grand Island, NY) was performed at 400 μg/ml. DSDh cell clones harboring replication-competent proviruses were maintained in the constant presence of neutralizing anti-SNV sera to prevent superinfection, as described by Dougherty and Temin (1986).

Transfection

Transfections were performed by the dimethyl sulfoxide/polybrene method (Kawai and Nishizawa, 1984).

Virus

All infections were performed using fresh virus stocks and polybrene as described by Burns and Temin (1994).

Isolation of doubly infected helper cell clones

Doubly infected helper cell clones containing pairs of vectors with and without runs of identical nucleotides were established by infecting cells with one virus and then superinfecting the same cells with the appropriate second virus (Fig. 2).

Recombination rate

Viruses were harvested from doubly resistant helper cell clones, diluted serially, and used to infect D-17 target cells (Fig. 2, step 3). The infected D-17 cells were placed under three separate drug selections in parallel (G418, hygromycin B, and G418 plus hygromycin B). Each drug selection was performed in replicates of two to six. Viral titers were determined by counting the drug-resistant colonies at the highest dilutions of virus. The recombination rates were calculated using the double resistant titer and the lower of the two single resistant titers as follows: $\text{Rate} = [2(\text{Double}^r \div \text{Lower Single}^r)] \div 1.029\ \text{kb} \div 1\ \text{cycle}$.

Cellular lysates

Cells from a confluent 100-mm tissue culture plate were lysed, treated with proteinase K, boiled, and frozen at −20°C as described by Burns and Temin (1994).

Polymerase chain reaction

Genomic DNAs from cell lysates were used as templates in PCR to amplify specifically the proviral DNA. For analysis of the Double^r step 3 cell clones, one round of PCR (two rounds, if needed) was performed using primers Neo1115 and 3288YH (Hu and Temin, 1992) in the first round and primers 32 and 33 in the final round. Primer sequences are as follows: (1) primer 32, 5′-CAGCCGAACTGTTCGCCAGGC-3′, and (2) primer 33, 5′-CCTTGCGGTCCGAATGGGCCG-3′.

For all first-round reactions, 50 μl of cellular lysate was amplified in a 100-μl reaction. Prior to the first round, reactions were heated at 94°C for 2 min and incubated at 72°C during the addition of enzyme (hot start). Five microliters was transferred into subsequent reactions, and all rounds consisted of 30 cycles. Reaction conditions varied for each set of primers according to the

melting temperatures and distances between the primers. Vent DNA polymerase (New England Biolabs) or *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD) was used for all amplifications according to the manufacturer's suggestions. The reaction mixture for round one was supplemented with 4.0 mM $MgSO_4$ when Vent polymerase was used. All samples were amplified on a Perkin–Elmer GeneAmp PCR System 9600.

DNA sequencing

Double-stranded plasmid DNA and PCR-amplified DNA were used as templates for sequencing. For direct sequencing of PCR-amplified DNA, three 100-μl reactions were combined and concentrated to a volume of 40 to 75 μl. Primers from PCR were removed by purification on CHROMA SPIN-400 columns (Clontech Laboratories, Inc., Palo Alto, CA). Sequencing was performed using the Sequenase DNA Sequencing Kit (United States Biochemical Corp., Cleveland, OH), and reactions were analyzed on a denaturing 6% polyacrylamide gel.

ACKNOWLEDGMENTS

We thank Dr. Howard Temin (deceased) and Dr. Norman Drinkwater for scientific discussions in the early phases of research. We thank Dr. Kimberly Perrine, Paul Jacques, Ingrid Hermanson, Adam Skepner, and Christine Downey for comments on the manuscript. We thank Joel Chaney, Dr. Valerie Shalin, Dr. Kathleen Beal, and Paul Jacques for statistical consultation. This work was supported by Public Health Service Grant CA72239 and by funds from McArdle Laboratory for Cancer Research, Wright State University, and the State of Ohio Research Challenge Grant.

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